CHAPTER 3

METHODOLOGY

1. Collection of human specimen

The collection of human specimen was approved by the Ethical Committee of the Faculty of Medicine, Thammasat University. All subjects participated in the study after giving written informed consents.

Human bone marrow was obtained from sternum or iliac crest of normal healthy volunteers of both sexes (age between 18-80 years). Subjects which have any clinical history of malignancy, metabolic disorder, or infectious disease, were excluded.

The samples of umbilical cord, Wharton's jelly, placenta and amnion were obtained from pregnanted women after normal deliveries at Siriraj Hospital, Mahidol University.

Peripheral blood samples were collected from healthy volunteer of both sexes (age between 18-80 years). Subjects which have any clinical history of malignancy, metabolic disorder, or infectious disease, were excluded.

2. Isolation and culture of MSCs from bone marrow

Fourty ml bone marrow by bone marrow aspiration and placed was collected from sternum or iliac crest of normal healthy volunteers (n=5) in 50 ml sterile tube (costar, corning, NY) containing 2 ml heparin (LEO 5,000 i.u./u.i./ml). To isolate mononuclear cells (MNCs) from bone marrow, the aspirate was diluted with equal volumn of phosphate-buffered saline (1X PBS) and carefully layered over Ficoll-Hypaque solution (GE Healthcare Bio-science AB, Sweden). After density gradient centrifugation at 100g for 30 min at 20°C, without breaking, MNCs were removed from the interphase layer (Fig 3.1) and washed twice with 1X PBS containing 100 U/ml penicillin and 100 μg/ml streptomycin. Cell numbers were determined using an automatic cell analyzer (Cell-DYN®1800, Abbott, Germany). Bone marrow-derived MNCs were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (GibcoBRL, NY) containing 2 mM L-

glutamine (GibcoBRL, NY), 10% fetal bovine serum (FBS)(BioWhittaker, USA), 100 U/ml penicillin and 100 μg/ml streptomycin at a density of $1X10^5$ cells/cm^2 in 25-cm^2 tissue culture flasks (Costar, Corning, NY). After 3 days incubation at 37°C in humidified atmosphere containing 5% carbon dioxide, non-adherent cells were removed and fresh medium was added to the flasks. Cultures were maintained in this condition with complete exchange of culture medium every 3-4 days. Culture flasks were observed continuously to get hold of developing colonies of adherent cells. After culture for 7-12 days when the cell density reach 90% confluence, they were passaged by incubation with 0.25% trypsin-EDTA (GibcoBRL, NY) and replaced at density at $1X10^4$ cell/cm^2 for further expansion. Some batchs of cultured cells were cryopreserved using freezing medium (90% FBS and 10% DMSO) and stored in liquid nitrogen for future use.

Figure 3.1 Bone marrow derived mononuclear cells at interphase layer.

3. Isolation and culture of MSCs from umbilical cords

Umbilical cords were obtained from pregnant women after normal delivery (gestational ages: 39-40 weeks, n=5, lengths: 2-4 cms) under aseptic conditions and were carried to the lab in sterile 1X PBS containing 100 U/ml penicillin/ 100 μg/ml streptomycin and processed within 4 h. The umbilical cords were rinsed with 1X PBS containing 100 U/ml penicillin/ 100 μg/ml streptomycin minced into small pieces (approximately 1-2 mm^3 in size) and then washed with 1X PBS containing 100 U/ml penicillin/ 100 μg/ml streptomycin. After centrifugation at 100g for 5 min, 5 ml 0.25% trypsin-EDTA (GibcoBRL, NY) was added and the tissues were incubated at 37°C with agitation for 30 min. At this stage, the cell suspension was centrifuged at 100g for 5 min, washed with 1X PBS containing 100 U/ml penicillin/ 100 μg/ml streptomycin and then resuspended in Dulbecco's Modified Eagle's Medium (DMEM) (GibcoBRL, NY) supplemented with 2 mM L-glutamine (GibcoBRL, NY), 10% FBS (BioWhittaker, USA), 100 U/ml penicillin/ 100 μg/ml streptomycin (GibcoBRL, NY). At this stage, the cells were seeded in noncoated 25-cm^2 tissue culture flasks (Costar, Corning, NY) (Fig. 3.2) and maintained at 37°C in a humidified atmosphere containing 5% CO_2, with a change of culture medium every 3-4 days. Culture flasks were observed continuously to get hold of developing colonies of adherent cells. Approximately 1-2 weeks later, when well-developed colonies of fibroblast-like cell appeared and the cell density reach 90% confluence, they were passaged by incubation with 0.25% trypsin-EDTA (GibcoBRL, NY) and replaced at density at $1X10^4$ cell/cm^2 for further expansion. Some batchs of cultured cells were cryopreserved using freezing medium (90% FBS and 10% DMSO) and stored in liquid nitrogen for future use.

Figure 3.2 Isolation and culture of MSCs from umbilical cord.

4. Isolation and culture of MSCs from Wharton's jelly

Umbilical cords (n=5, gestational ages: 39-40 weeks, lengths: 5-10 cm) were obtained after birth and stored in 1X PBS containing 100 U/ml penicillin/ 100 µg/ml streptomycin before processing to obtain mesenchymal cells. After removal of blood vessels, the mesenchymal tissue was scraped off from the Wharton's jelly with a scalpel. After that the tissue was washed with 1X PBS, containing 100 U/ml penicillin/ 100 µg/ml streptomycin and then minced into small pieces (approximately 1-2 mm^3). The tissue was then treated with 0.5% trypsin-EDTA (GibcoBRL, NY) for 30 min at 37°C with agitation. After, washing with 1X PBS containing 100 U/ml penicillin/ 100 µg/ml streptomycin, the pellet was resuspended in Dulbecco's Modified Eagle's Medium (DMEM) (GibcoBRL, NY) supplemented with 2 mM L-glutamine (GibcoBRL, NY), 10% FBS (BioWhittaker, USA), 100 U/ml penicillin/ 100 µg/ml streptomycin (GibcoBRL, NY) and seeded in noncoated 25-cm^2 tissue culture flasks (Costar, Corning, NY) (Fig. 3.3). Culture was maintained at 37°C in a humidified atmosphere containing 5% CO_2, with a change of culture medium every 3-4 days. Culture flasks were observed continuously to get hold of developing colonies of adherent cells. Approximately 1-2 weeks later, when well-developed colonies of fibroblast-like cell appeared and the cell density reach 90% confluence, they were passaged by incubation with 0.25% trypsin-EDTA (GibcoBRL, NY) and replaced at density at $1X10^4$ cell/cm^2 for further expansion. Some batchs of cultured cells were cryopreserved using freezing medium (90% FBS and 10% DMSO) and stored in liquid nitrogen for future use.

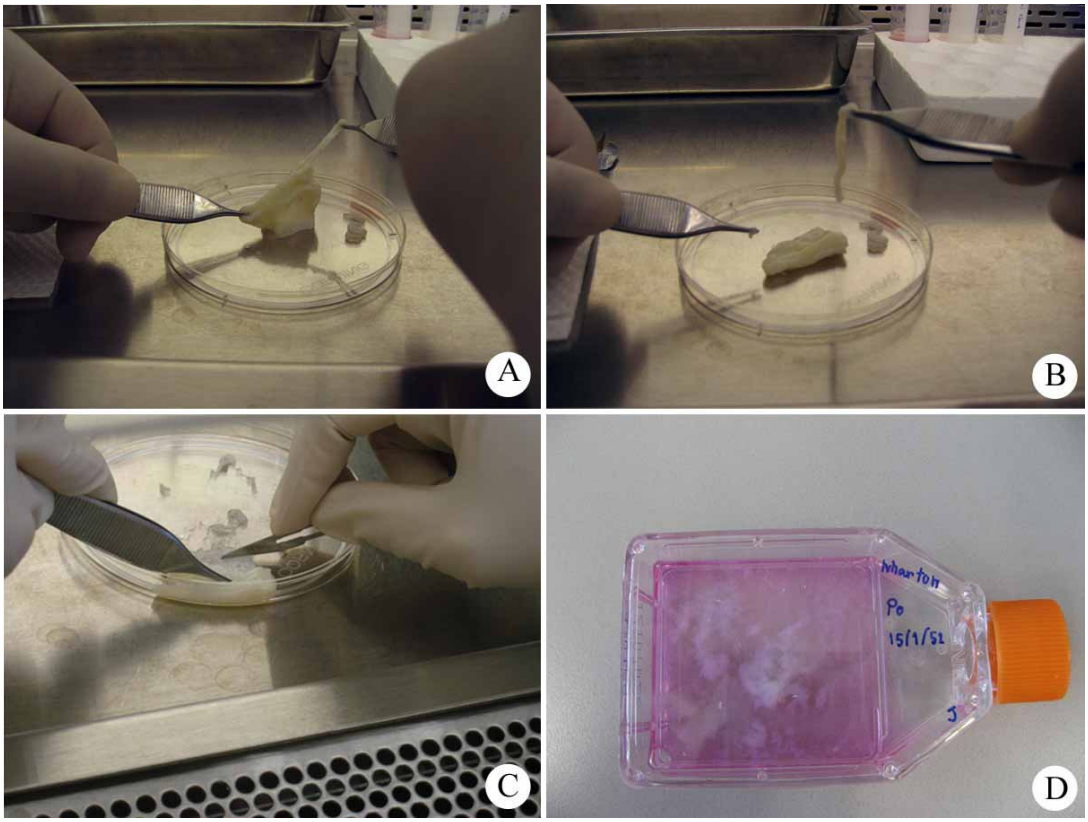

Figure 3.3 Isolation and culture of MSCs from Wharton's jelly.

5. Isolation and culture of MSCs from placenta

Decidua basalis tissue (n=5, gestational ages: 39-40 weeks, 3x3x1 cm) was macroscopically dissected from the central region of the maternal-facing surface of the placenta under aseptic conditions and were carried to the lab in 1X PBS containing 100 U/ml penicillin/ 100 μg/ml streptomycin. In a biohazard cabinet, the placental tissue was minced into small pieces (approximately 1-2 mm^3) and extensively washed with 1X PBS containing 100 U/ml penicillin/ 100 μg/ml streptomycin. Subsequently, the tissue was digested with 0.5% trypsin-EDTA (GibcoBRL, NY) for 30 min at 37°C with agitation. The pellet was washed with 1X PBS containing 100 U/ml penicillin/ 100 μg/ml streptomycin and then centrifuged at 100 g for 5 min. The pellet were then resuspended in Dulbecco's Modified Eagle's Medium (DMEM) (GibcoBRL, NY) supplement with 2 mM L-glutamine (GibcoBRL, NY), 10% FBS (BioWhittaker, USA), 100 U/ml penicillin/ 100 μg/ml streptomycin (GibcoBRL, NY) and plated into noncoated 25-cm^2 tissue culture flasks (Costar, Corning, NY) (Fig. 3.4). Culture was maintained at 37°C in a humidified atmosphere containing 5% CO_2, with a change of culture medium every 3-4 days. Culture flasks were observed continuously to get hold of developing colonies of adherent cells. Approximately 2-3 weeks later, when well-developed colonies of fibroblast-like cell appeared and the cell density reach 90% confluence, they were passaged by incubation with 0.25% trypsin-EDTA (GibcoBRL, NY) and replaced at density at $1X10^4$ cell/cm^2 for further expansion. Some batchs of cultured cells were cryopreserved using freezing medium (90% FBS and 10% DMSO) and stored in liquid nitrogen for future use.

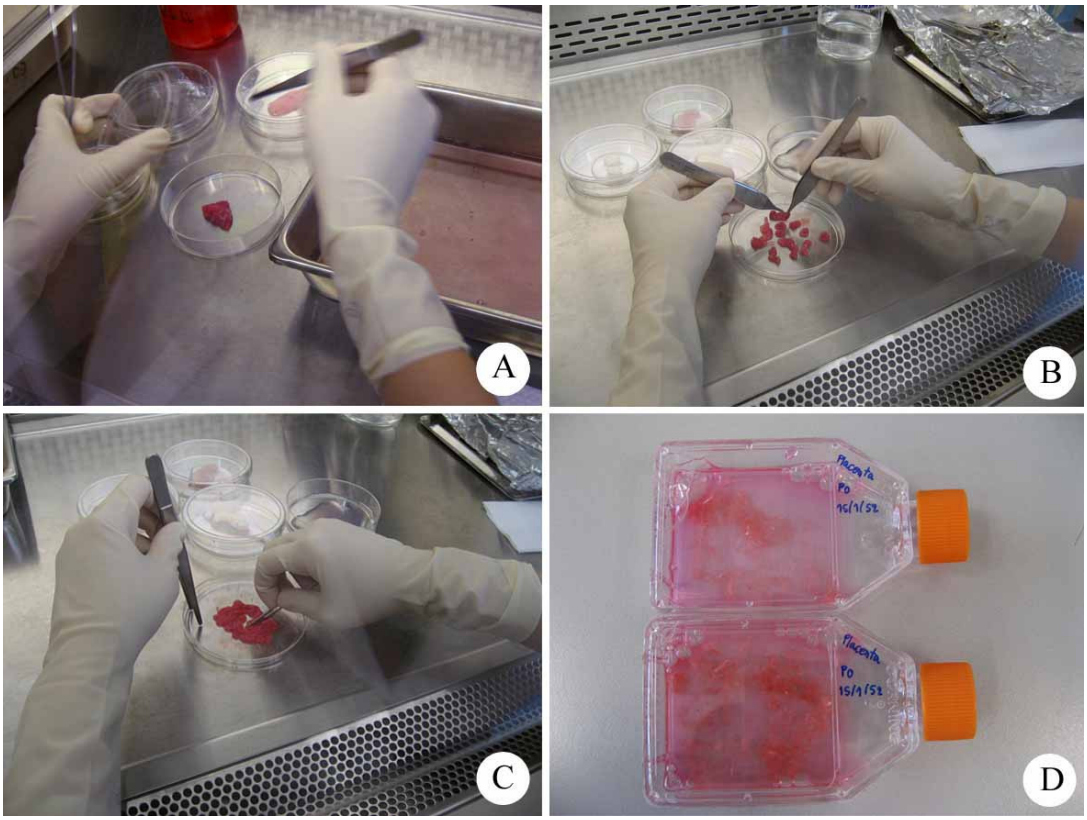

Figure 3.4 Isolation and culture of MSCs from placenta.

6. Isolation and culture of MSCs from amnion

Term amnion (n=5, gestational ages: 39-40 weeks, diameter~ 5 cm) was obtained by mechanically peeled off from chorion under aseptic conditions. It was carried to the lab in filtered phosphate buffered saline (1X PBS) containing 100 U/ml penicillin/ 100 μg/ml streptomycin and processed within 4 h. The amnion was rinsed with 1X PBS containing 100 U/ml penicillin/ 100 μg/ml streptomycin and minced into small pieces (approximately 1-2 mm^3 in size). The tissue was then treated with 0.5% trypsin-EDTA (GibcoBRL, NY) for 30 min at 37°C with agitation. After, washing with 1X PBS containing 100 U/ml penicillin/ 100 μg/ml streptomycin, the pellet was resuspended in Dulbecco's Modified Eagle's Medium (DMEM) (GibcoBRL, NY) supplemented with 2 mM L-glutamine (GibcoBRL, NY), 10% FBS (BioWhittaker, USA), 100 U/ml penicillin/ 100 μg/ml streptomycin (GibcoBRL, NY) and seeded in noncoated 25-cm^2 tissue culture flasks (Costar, Corning, NY) (Fig. 3.5). Culture was maintained at 37°C in a humidified atmosphere containing 5% CO_2, with a change of culture medium every 3-4 days. Culture flasks were observed continuously to get hold of developing colonies of adherent cells. Approximately 2-3 weeks later, when well-developed colonies of fibroblast-like cell appeared and the cell density reach 90% confluence, they were passaged by incubation with 0.25% trypsin-EDTA (GibcoBRL, NY) and replaced at density at $1X10^4$ cell/cm^2 for further expansion. Some batchs of cultured cells were cryopreserved using freezing medium (90% FBS and 10% DMSO) and stored in liquid nitrogen for future use.

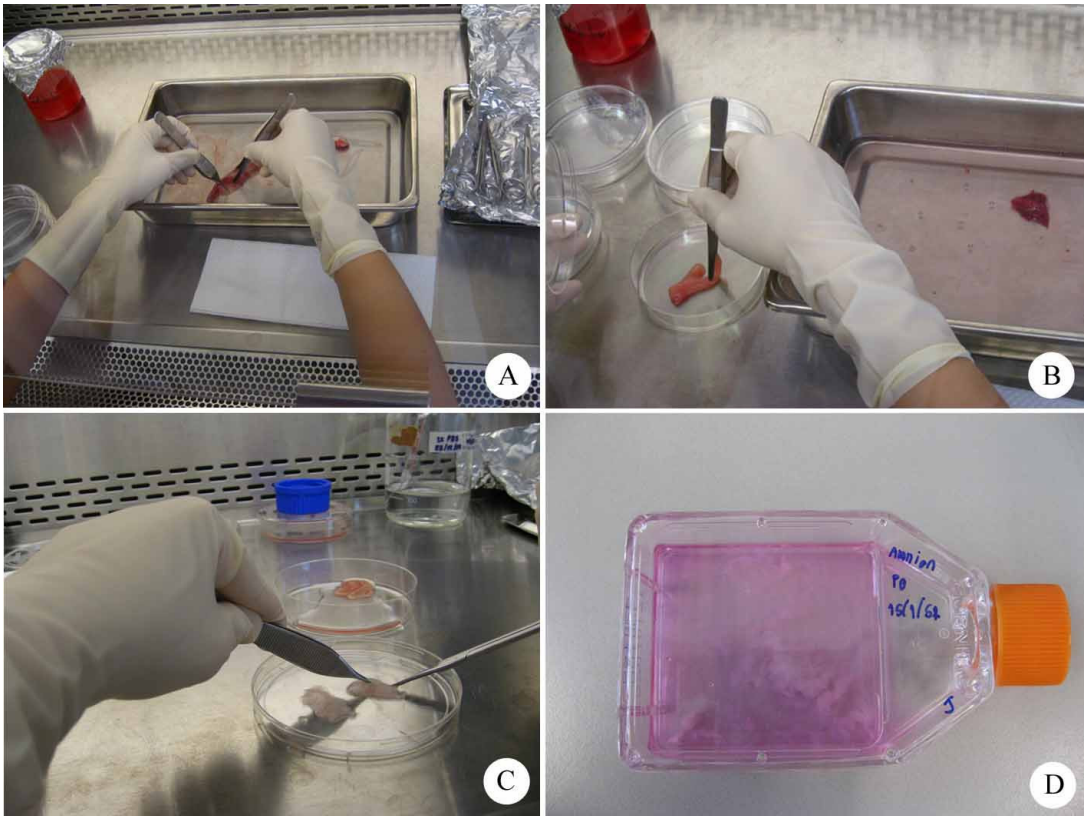

Figure 3.5 Isolation and culture of MSCs from amnion.

7. Immunophenotyping of culture cells

The culture cells were immunophenotypically characterized using flow cytometer (FACScalibur™, Becton Dickinson, USA). Primary cultures from bone marrow, umbilical cord, Wharton's jelly, placenta and amnion (passage 2-5) were trypsinized with 0.25% trypsin-EDTA (GibcoBRL, NY), washed twice with 1X PBS and fixed with 1% paraformadehyde in 1X PBS for 15 min. After washing twice with 1X PBS, $4X10^5$ cells were resuspended in 50 µl 1X PBS and incubated with 10 µl of fluorescein isothiocyanate (FITC) or phycoerythrin (PE) - conjugated antibodies against CD34 (BD Bioscience, USA), CD45 (BD Bioscience, USA), CD73 (BD Bioscience, USA), CD90 (AbD Serotec, USA), CD105 (Ab Serotec, USA) and CD106 (AbD Serotec, USA) for 30 min at 4°C in the dark. The positive cells were then identified by comparison with isotypic controls [FITC-conjugated mouse immunoglobulin G1 (IgG1) and PE-conjugated mouse immunoglobulin G2a (IgG2a)]. At least ten thousand labeled cells were acquired and analyzed using FACScalibur™ with CellQuest® solfware (Becton Dickinson, USA).

8. Proliferation studies

For the assessment of growth characteristics of MSCs derived from umbilical cord, Wharton's jelly, placenta and amnion in comparison to bone marrow derived MSCs, $6x10^3$ culture-expanded MSCs (passage 2-5) were seeded in 24-well cell culture plate (Costar, Corning, NY) containing 1 ml Dulbecco's Modified Eagle's Medium (DMEM) (GibcoBRL, NY) supplemented with 2 mM L-glutamine (GibcoBRL, NY), 10% FBS (BioWhittaker, USA), 100 U/ml penicillin and 100 µg/ml streptomycin (GibcoBRL, NY). The cells from one well were then harvested at culture day 2, 4, 6 and 8 to determine cell number by hemocytometer. The mean of the cell counts was calculated and plotted against culture time to generate a growth curve.

9. Adipogenic differentiation testing

The adipogenic differentiation potential of umbilical cord, Wharton's jelly, placenta, amnion and bone marrow-derived MSCs were examined using passage 3-cells. Culture-expanded MSCs from umbilical cord, Wharton's jelly, placenta, amnion and bone marrow were resuspended in 1.5 ml NH AdipoDiff Medium (MACS® Media, USA) and plated at a density of $8X10^3$ cells/cm^2 in 6-well plate for adipogenic differentiation. Cells maintain in Dulbecco's Modified Eagle's Medium (DMEM) (GibcoBRL, NY) supplemented with 2 mM L-glutamine (GibcoBRL, NY), 10% FBS (BioWhittaker, USA), 100 U/ml penicillin/ 100 μg/ml streptomycin (GibcoBRL, NY) served as controls. The cells were cultured at 37°C in with 5% CO_2 humiditied atmosphere with complete exchange of medium every 3 days. After 3 weeks of culture, cells were washed with 1X PBS and fixed with 40% formalin vapor for 10 min at room temperature. At this stage, the cells were washed twice with distilled water and stained with 0.5% Oil Red-O (Sigma-Aldrich, USA) in 60% isopropanol for 20 min with agitation at room temperature. Thereafter, the cells were washed twice with distilled water and examined under inverted microscope (Olympus, CKX41).

10. Osteogenic differentiation testing

The osteogenic differentiation potential of umbilical cord, Wharton's jelly, placenta, amnion and bone marrow-derived MSCs were examined using passage 3-cells. Culture-expanded MSCs (passage 3) from umbilical cord, Wharton's jelly, placenta, amnion and bone marrow were seeded at a density of $5X10^3$ cells/cm^2 , resuspended in 1.5 ml in NH OsteoDiff Media (MACS® Media, USA) into 6-well plate (Costar, Corning, USA) for osteogenic differentiation and cells were culture at 37°C in an incubator with 5% CO_2 at saturating humidity. The medium was replaced every 3 days. After 10 days of culture, cells were washed with 1X PBS and fixed with 10% formalin-methanol for 5 min at -20^{o}C. To visualize osteogenic differentiation, cells were stained for alkaline phosphatase (AP) activity.

For AP expression, cells were washed with 1X PBS and subsequently incubated for 10 min with substrate solution (1 mg/ml sodium naphthyl, 1 mg/ml

fast blue RR acid in propanidial buffer pH 9.75), resulting in the formation of a purple reaction product. The positive cells were observed under inverted microscope (Olympus, CKX41).

Control cultures without the differentiation stimuli were carried out in pararell to the experiments and stained in the same manner.

11. Mixed lymphocyte reaction assays (MLR assays)

Preparation of peripheral blood mononuclear cells

50 ml peripheral blood (one serve as a stimulator, another as a responder) was obtained from median cubital vein of two allogeneic healthy volunteers (n=5) and placed in 50 ml Falcon tube (Becton Dickinson, USA) containing 2 ml heparin (LEO 5,000 i.u./u.i./ml). To isolate mononuclear cells (MNCs), the blood was diluted with equal volumn of phosphate-buffered saline (1X PBS) and carefully layered over Ficoll-Hypaque solution (GE Healthcare Bio-science AB, Sweden). After density gradient centrifugation at 100g for 30 min at 20°C, without break, MNCs were harvested from the interphase layer and washed twice with 1X PBS containing 100 U/ml penicillin and 100 µg/ml streptomycin. MNCs number were determined using an automatic cell analyzer (Cell-DYN®1800, Abbott, Germany).

Labeling Responder cells with CFSE

Peripheral blood mononuclear cells (PB-MNCs) from responder were labeled with 0.5 µM 5(6)-carboxyfluorescein diacetate *N*-succinimidyl ester (CFSE; Sigma) for 15 min at 37°C with continuous shake. After washing the cells with RPMI 1640 medium (GibcoBRL, NY) supplemented with 10% FBS (BioWhittaker, USA), 2 mM L-glutamine (GibcoBRL, NY), 100 U/ml penicillin/ 100 µg/ml streptomycin (GibcoBRL, NY) for 3 times, the labeled cells were then resuspended in the same medium and kept at 4^{o}C in the dark until required for furture experiment.

Irradiating stimulator cells

PB-MNCs from stimulator were irradiated at 3000 rad. for 2.5 min. After washing the irradiated cells with RPMI 1640 medium (GibcoBRL, NY) supplemented with 10% FBS (BioWhittaker, USA)), 2 mM L-glutamine (GibcoBRL, NY), 100 U/ml penicillin/ 100 µg/ml streptomycin (GibcoBRL, NY) for 3 times, the cells were then resuspended in the same medium and kept at room temperature until required for furture experiment.

Mixed lymphocyte reaction assay

To test the potential of MSCs in suppressing proliferation of activated T-lymphocyte, a mix lymphocyte reaction (MLR) assay was performed using peripheral blood MNCs (PB-MNCs) obtained from two allogenic volunteers (n=5 pairs). CSFE-labeled responder's PB-MNCs and irradiated PB-MNCs from an allogeneic donor were co-cultured at a ratio of 1:1 in 24-well flat-bottomed plate (Costar, Corning, USA) containing RPMI-1640 medium (GibcoBRL, NY) supplemented with 10%FBS (BioWhittaker, USA)), 2 mM L-glutamine (GibcoBRL, NY), 100 U/ml penicillin/ 100 µg/ml streptomycin (GibcoBRL, NY). The effectors cells (MSCs derived from umbilical cord, Wharton's jelly, placenta, amnion and bone marrow) were also plated into the same wells. The positive control was CSFE-labeled responder PB-MNCs and irradiated PB-MNCs from an allogeneic donor without MSCs added. In assays of mitogen-induced proliferation, 1 x 10^5 PB-MNCs were cultured with phytohemagglutinin (PHA; 2 µg/ml) without the presence of MSCs. After 5 days of MLR culture, the MNCs were harvested and washed twice with 1X PBS containing 1% FBS (BioWhittaker, USA). MNCs were resuspended in 1X PBS containing 1% FBS (BioWhittaker, USA). 7-aminoactinomycin D (7-ADD) staining was used to exclude dead cells, and analysis of cell division was performed using flow cytometer (FACScalibur^{TM}, Becton Dickinson, USA) and Cell Quest software. All experiments were performed at least in triplicate. The data was presented as a proliferative index which was calculated by the following formula:

$$\text{Proliferative index} = \left(\frac{A}{A+B} \right) \times 100$$

A= number of cell division (R5)

B= number of cell in original population (R3)

12. Statistical analysis

Unless otherwise stated, data are presented as mean ± standard error of mean (SEM). The ANOVA test was used to assess the significance of differences between observed data. *P*-value of less than 0.05 was considered to be statistically significant.